AD_____

Award Number: DAMD17-98-1-8514

TITLE: Oral Contraceptives Use by Young Women Reduces Peak Bone Mass

PRINCIPAL INVESTIGATOR: Thomas Register, Ph.D.

CONTRACTING ORGANIZATION: Bowman Gray School of Medicine
Winston-Salem, North Carolina 27157

REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Annual (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
Oral Contraceptives Use by Young Women Reduces Peak Bone Mass

5. FUNDING NUMBERS
DAMD17-98-1-8514

6. AUTHOR(S)
Thomas Register, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wake Forest University School of Medicine
Winston-Salem, North Carolina 27157

E-MAIL:
register@wfubmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The goal of these studies was to determine the role that hypoandrogenemia plays in the effects of oral contraceptives (OC) on bone metabolism and peak bone mass (PBM) in young female rats. Adolescent/young adult Sprague-Dawley rats were treated with 1) placebo, 2) OC, 3) OC supplemented with an androgen (methyltestosterone), or 4) an anti-androgen (bicalutamide) to determine the potential role that suppression of androgens plays on bone metabolism, bone architecture, and the attainment of PBM. Our specific aims were to determine:

1. If oral contraceptive steroid (OC) treatment leads to decreased peak bone mass in young intact female rats. *Findings: OC use decreased the peak bone mass of young intact female rats.*
2. If the addition of a non-aromatizable androgenic steroid to OCs prevents the detrimental effects of OC use on peak bone mass. *Findings: The non-aromatizable androgenic steroid did not prevent the adverse effects of OCs to the growing skeleton of young rats at the dose used.*
3. If anti-androgen treatment mimics the effect of OC use on peak bone mass. *Findings: The anti-androgen used did not mimic the adverse effect of OCs on the growing skeleton of young rats.*

14. SUBJECT TERMS	15. NUMBER OF PAGES
osteoporosis, peak bone mass, oral contraceptives, androgens	20
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The central hypothesis underlying the proposed study was that oral contraceptive (OC) treatment of adolescent and young adult females causes an abnormal depression of circulating androgens which results in a depression of bone gain during this critical period. The end result may be a reduction in peak bone mass and an increased risk of stress fractures and osteoporosis. Similar results might be observed by suppression of androgen activity in intact animals in the absence of OC therapy. Conversely, supplementation of OC-treated females with an androgen may result in restoration of normal bone maturation. The overall goal of the proposed study was to determine the role that hypoandrogenemia plays in the effects of OC on bone metabolism and on peak bone mass in young female rats. For these studies, we used Sprague-Dawley rats, a well-characterized animal model of ovarian hormone effects on bone metabolism. These animals were examined while in the adolescent and young adult age range. We treated intact animals with 1) Placebo, 2) OC, 3) OC supplemented with an androgen (methyltestosterone), or 4) Anti-androgen therapy (bicalutamide) to determine the potential role that suppression of androgens plays on bone metabolism, bone architecture, and the attainment of PBM.

BODY

Personnel

- Dr. Manuel Jayo, Senior Pathologist with Pathology Associates International and adjunct Associate Professor of Pathology at Wake Forest University School of Medicine.
-
- Dr. Erni Sulistiawati, an Indonesian D.V.M. and a Ph.D. candidate enrolled at the Institut Pertanian Bogor (IPB). October 1, 1998 - Aug 30, 1990. Dr. Sulistiawati's mentor in Indonesia was Dr. Dondin Sajuthi.

- Dr. Uriel Blas-Machado, post-doctoral fellow. September 1, 1998 to June 11, 1999. Dr. Blas-Machado's salary was supported by a Training Grant from the NCRR, NIH.

- Technical assistance was provided by Mrs. Pam Louderback, Mr. Sam E. Rankin, and Mr. Gerald Perry.

Experimental Procedures and Outcomes

- Seven rats (10% of total approved by the institutional Animal Care and Use Committee [ACUC]) were used to conduct the pilot project (TABLE 1). This project allowed us to test the palatability and the feasibility of procedures (sedation, bleeding, densitometry, etc) to be conducted in the live animals.

TABLE 1. PILOT PROJECT

Exp Time	*Week*	*Age (days)*	*Comment*	*Date*
-1	1	63		26-Oct-98
Start diet	0	70	DXA 1	02-Nov-98
1	3	77		09-Nov-98
2	4	84	DXA 2	16-Nov-98

Based on our previous work with non-human primates (Register et al., 1997), the food consumption and body weight gains during the pilot project, no additional palatability issues were considered and the go ahead for the proposed experiment was given.

- As part of the annual review, on October 19, 1998, the ACUC Protocol A97-147 was approved for extension until October 20, 1999.

- Semipurified food (with hormones) was prepared (Table 2) and keep frozen until ready to use. Once open, it was kept refrigerated.

Table 2. Semi-purified diet, designed to contain no isoflavones. Each 100 g of semi-purified high-fat diet contained the following products.

Food	*(g)*
Casein, USP	10.5
Lactalbumin	10.0
Dextrin	30.6
Sucrose	28.0
Alphacel	10.0
Lard	5.20
Safflower Oil (linoleic)	1.00
Choline Bitartrate	0.20
Vitamin Mixture, AIN-76A	1.00
Mineral Mix, AIN-76	3.50

- In contrast to our pilot information from rats and our previous monkey data, we found the rats were not eating as expected (Table 3). After review, feed was to be produced every other week to maintain palatability. The differences in total consumption were dramatic, on average 3 to 4 g of food per day were not consumed by the OC and OC+MT groups (Table 3).

Table 3. Average (AVE, g) feed consumption per day during the experiment

Group	**AVE ± SD**
Control	20.02 ± 1.94
OC	16.79 ± 4.80
OC+MT	17.09 ± 5.16
Cas	19.35 ± 2.09

- The average feed consumption varied with the contraceptive schedule (3 days on and 1 day off, to mimic a woman's pill cycle) as shown in Table 4:

Table 4. Average (g) feed consumption per day-cycle

Group	**Day 1**	**Day 2**	**Day 3**	**Day 4 (*NO STEROIDS*)**
Control	19.71	20.18	19.79	20.42
OC	12.49	15.03	15.39	24.29
OC+MT	11.96	15.41	16.46	24.53
Cas	18.91	19.47	18.99	20.03

- Two fluoroscein bone labels were ordered and given (demeclocycline and calcein) prior to necropsy.

- Necropsies and collection of tissues were carried out in March 1999. Type and number of tissues per animal collected processed, sectioned, stained (H&E), and histologically evaluated included: ovaries (2), uterus and horns (2), vagina, cervix and urinary bladder (2), liver lobes (3), spleen and kidneys (3), adrenal glands (2), thyroids, thymus, and pancreas (3), heart (2), lungs (2), brain (2), mammary gland (2), pituitary (1), left femur (1) and L2 vertebra (1).

- At necropsy, the right tibia and L3 vertebrae were collected, the soft tissue cleaned, and the bones placed in dark-brown stained 30 ml glass bottles containing 70% alcohol (ETOH). The right tibia's tuberosity was shaved with a sharp scalpel blade for proper fixation and the dorsal arches of the lumbar L3 vertebra removed.

- Bones were packaged and sent to Pathology Associates International (PAI) in Frederick, MD for plastic bone histologic processing. *Bone Histomorphometry:* PAI processed, embedded in methyl methacrylate (MMA), and sectioned at 5-10 μm, and mounted unstained or stained sections of bone with modified tetrachrome with Von Kossa method. *Standard histomorphometry*: The abbreviations used were based on the ASBMR standard nomenclature (1). Structural and dynamic parameters were measured.

- Soft and hard tissues were fixed, processed, embedded, section and stained for evaluations by Drs. Jayo and Blas-Machado.

Ex vivo primal and distal pQCT scanning of tibia

Methods

After necropsy, the right tibia was kept frozen at −20°C until scanned using peripheral quantitative computed tomography (pQCT). The Norland Stratec XCT960 pQCT Bone Densitometer (Ft. Atkinson, WI) was used for pQCT measurements. Although methodology differed slightly from other reports, precision was similar to that previously reported (Gasser 1995, Sato 1997). A voxel size of 0.148 mm and a threshold for cortical bone of 500 was selected throughout the scans (Contour Mode 1, Peel mode 2, Cortical mode 4). Scans were taken at the proximal (metaphyseal and cancellous rich) and distal (primarily cortical) portions of the tibia. Based on previous reports and histological evaluations, pQCT scans were taken for proximal tibia at a constant 5 mm distance from the knee joint. Distal tibia evaluations were taken at a constant 1 mm proximal to the fibulo-tibial junction. For both sites, measurements included Cancellous Bone Mineral Content (Cn.BMC, in mg/mm [trab_cnt]), Cancellous Bone Mineral Density (Cn.BMD, in mg/mL [trab_dn]), Cancellous Bone Area, (Cn.B.Ar, in mm^2, [trab_a]), Cortical Bone Mineral Content (Ct. BMC, in mg/mm, [crt_cnt]), Cortical Bone Mineral Density (Ct.BMD, in mg/mL, [crt_den]), Cortical Bone Area, (Ct.B.Ar, in mm^2, [crt_a]), Cortical Thickness (Ct.Th., mm, [crt_thk]), Periosteal perimeter (Ps.Pm, mm, [peri_c]), Endosteal Perimeter (Ec.Pm, mm, [endo_c]), Polar Moment of Inertia (P.M.I., mm^4, [ip_cm_w]), and Moment of Resistance or the (P.M.R., mm^3, [rp_cm_w]).

Statistics

All QCT raw data is expressed as mean ± SEM (Table 5). All statistical analyses were conducted using version 7.0 BMDP Statistical Software (Los Angeles, CA). Data was subjected to one-way analysis of variance (ANOVA) and post hoc pairwise comparisons utilizing Tukey's test. The letter symbol in all tables and graphs indicate the level of significance compared to Control animals ($^a p<0.05$; $^b p<0.01$).

Table 5. pQCT measurements taken from the right proximal tibia of young female rats at a constant 5 mm distal site from the joint space.

Parameter	*Control*	*OC*	*OC+MT*	*Casodex*	*p-value*
N	14	14	14	12	x
Cn.BMC	1.10 ± 0.15	1.46 ± 0.08	**1.61 ± 0.06[b]**	1.11 ± 0.14	0.0023
Cn.BMD	308 ± 9.04	**270 ± 9.77[a]**	**254 ± 10.8[b]**	305 ± 6.40	0.0002
Cn.B.Ar	3.72 ± 0.55	5.50 ± 0.39	6.46 ± 0.32	3.68 ± 0.48	0.0000
Ct. BMC	9.65 ± 0.25	**7.74 ± 0.19[b]**	**7.56 ± 0.23[b]**	9.34 ± 0.23	0.0000
Ct.BMD	922 ± 13.31	920 ± 9.56	917 ± 10.67	909 ± 19.30	NS
Ct.B.Ar	10.5 ± 0.33	**8.42 ± 0.24[b]**	**8.24 ± 0.20[b]**	10.3 ± 0.39	0.0000
Ct.Th	0.78 ± 0.03	**0.65 ± 0.01[b]**	**0.63 ± 0.02[b]**	0.77 ± 0.02	0.0000
Ps.Pm	15.9 ± 0.22	**14.9 ± 0.22[b]**	**15.0 ± 0.16[a]**	15.9 ± 0.26	0.0012
Ec.Pm	11.0 ± 0.26	10.8 ± 0.20	11.1 ± 0.17	11.0 ± 0.24	NS
P.M.I.	36.7 ± 1.29	**27.6 ± 1.26[b]**	**27.3 ± 1.05[b]**	35.8 ± 1.31	0.0000
P.M.R	11.3 ± 0.34	**8.90 ± 0.36[b]**	**8.78 ± 0.33[b]**	11.1 ± 0.41	0.0000

Results

The QCT-derived parameters measured at the distal tibia (primarily cortical bone) were not significantly different among groups (data not shown). However, significant differences between Control vs OC or OC+MT treated animals were detected at the proximal tibia in both cortical and cancellous parameters (Table 1). None of the measurements were significantly different between Control and Casodex groups. *

Conclusions

OC use in growing rats, at a dose which corresponds to a 25% lower dose than that recommended for contraception in women, caused bone deficits at the proximal tibia compared to Control animals. This bone deficit was not prevented by OC supplemented with the androgen methyltestosterone. Surprisingly, and in contrast to previous reports (Lea et al., 1996), the nonsteroidal anti-androgen bicalutamide (Casodex) ingestion in growing rats did not cause significant bone changes compared to Control rats. Dose and route of administration were different in the two studies, as Lea et al. (1996) administered Casodex SQ daily ti rats at 20 mg/kg/day for 21 days (420 mg total) while in the present study Casodex was given orally at a dose corresponding to a human dose of 50 mg/day (0.89 mg/100 g of BW in the rat) for 105 days.

Table 6. **Histomorphometric measurements for the distal femur metaphysis. Note: The total of distal femur metaphysis (bone + marrow) was identical for all groups (3.73 mm^2).**

Parameter	*Group*	*Mean*		*SEM*	*P-value*
BV	OC	0.94		0.11	0.000
	Control	1.22	0.11		
	OC+MT	0.70	0.07		
	Casodex	1.30	0.13		
BS	OC	24.63		1.75	0.001
	Control	28.65	1.13		
	OC+MT	21.20	1.27		
	Casodex	30.34	2.23		
BV/TV	OC	25.24		2.84	0.000
	Control	32.68	2.96		
	OC+MT	18.70	1.77		
	Casodex	34.83	3.37		
Tb.Th.	OC	57.25		3.25	0.005
	Control	65.07	3.89		
	OC+MT	50.44	2.24		
	Casodex	65.05	3.26		
Tb.N.	OC	4.20		0.30	0.001
	Control	4.89	0.19		
	OC+MT	3.62	0.22		
	Casodex	5.18	0.38		
Tb.Sp.	OC	204.75		29.72	0.059
	Control	143.93	12.51		
	OC+MT	240.16	20.37		
	Casodex	158.23	42.43		

Soft tissue histologic evaluations

Ovaries Ovaries were evaluated by counting the number of primary, growing, and antral follicles. Corpora lutea (CL) were counted and classified into atretic CL, hemorrhagic CL, and mature CL.

Table 7.

Primary (ANOVA p=0.260)

	OC	Control	OC+MT	Casodex
N	13	14	14	12
Mean	31.615	26.000	35.000	21.833
STD	17.868	19.896	20.840	10.338
SEM	4.956	5.317	5.570	2.984
Min	82.000	69.000	74.000	35.000
Max	11.000	4.000	5.000	6.000

Growing (ANOVA p=0.312)

	OC	Control	OC+MT	Casodex
N	13	14	14	12
Mean	7.000	4.786	6.214	4.417
STD	3.851	3.043	3.641	3.397
SEM	1.068	0.813	0.973	0.981
Min	14.000	9.000	15.000	11.000
Max	3.000	0.000	2.000	0.000

Antral (ANOVA p=0.448)

	OC	Control	OC+MT	Casodex
N	13	14	14	12
Mean	12.077	13.143	9.357	11.750
STD	5.283	7.833	5.733	5.895
SEM	1.465	2.094	1.532	1.702
Min	22.000	26.000	19.000	24.000
Max	5.000	4.000	0.000	1.000

Atretic (ANOVA p=0.823)

	OC	Control	OC+MT	Casodex
N	13	14	14	12
Mean	12.077	13.143	9.357	11.750
STD	5.283	7.833	5.733	5.895
SEM	1.465	2.094	1.532	1.702
Min	22.000	26.000	19.000	24.000
Max	5.000	4.000	0.000	1.000

Bone Histomorphometry

Methods

The tibial length was measured with a caliper, and the tibia was cross-sectioned one mm above the tibio-fibular junction to obtain a cortical sample. Then the bones were processed and embedded in methyl methacrylate (MMA). The proximal tibia sample and the L3 vertebrae were sectioned (at 5-10 μm) with a macrotome and mounted for 2 unstained or 2 stained (modified tetrachrome with Von Kossa method and Toludine blue with TRAP). Two sections of the cortical tibia sample were ground (approximately 25 μm), one was left unstained and the other stained with modified tetrachrome with Von Kossa. Histomorphometry was conducted using established procedure and a True Color-98 Bioquant System (R&M Biometrics, Nashville, TN). The abbreviations used are based on the ASBMR standard nomenclature.[2]

Cortical Measurements at Tibio-Fibular Junction Structural and dynamic parameters were derived separately for periosteal (Ps), cancellous (Cn), cortical (Ct), and endosteal or endocortical (Ec) bone envelopes. For each tibia, one mm above the fibular attachment, the following parameters were measured and/or derived: average cortical thickness (Ave.Ct.Th, μm), tissue volume (TV, μm^2), periosteal perimeter (Pr.P, μm), core or marrow + Cn bone volume (Core.V, μm^2), endocortical perimeter (Ec.P, μm), cancellous bone volume (Cn.BV, μm^2), cancellous bone perimeter (Cn.P, μm), hole or cortical porosity volume and perimeter (HV and HP, μm^2 and μm respectively), cortical bone volume (Ct.BV, μm^2), marrow volume (Mw.V, μm^2), percent Ct bone (Ct.BV/TV, %), percent Cn bone (Cn.BV/TV, %), periosteal single labeled surface perimeter (Ps.sLS, μm), periosteal double labeled surface perimeter (Ps.dLS, μm), periosteal inter-label distance (Ps.ILD, μm), periosteal mineralizing surface (Ps.MS/BS, %), periosteal mineral apposition rate (Ps.MAR, μm/day), periosteal bone formation rate (Ps.BFR/BS, mm^3/mm^2/day), endocortical single labeled perimeter (Ec.sLS, μm), endocortical double labeled surface (Ec.dLS, μm), endocortical inter-label distance (Ec.ILD, μm), endocortical mineralizing surface (Ec.MS/BS, %), endocortical mineral apposition rate (Ec.MAR, μm/day), and endocortical bone formation rate (Ec.BFR/BS, mm^3/mm^2/day).

Cancellous Measurements at Proximal Tibia Structural and dynamic parameters were measured and/or derived for cancellous (Cn, trabecular) bone. Although the Cn bone may be affected by growth rates, measurement was taken at a standard area of approximately 2-3 mm^2 of bone tissue at least 1.0 mm away from the growth plate to exclude the primary spongiosa. For each proximal tibia, the bone and marrow tissue volume (TV, μm^2), bone tissue area (BA, μm^2), bone surface perimeter (BS, μm), Cn bone volume (BV, μm^2), percent Cn bone (BV/TV, %), trabecular bone thickness (Tb.Th, μm), trabecular separation (μm), trabecular number (#/mm), single labeled bone surface perimeter (sLS, μm), double labeled bone surface perimeter (dLS, μm), not labeled bone surface (nL.S, μm), mean inter-label distance (MILD, μm), mineralizing surface (MS/BS, %), periosteal mineral apposition rate (Ps.MAR, μm/day), bone formation rate surface referent (BFR/BS, mm^3/mm^2/day),), bone formation rate volume referent (BFR/BV, %/yr), and bone formation rate total tissue volume referent (BFR/TV, %/yr).

Cn Measurements of L3 Vertebra Similar to the proximal tibia, structural parameters were measured and/or derived for cancellous (Cn, trabecular) bone. For each vertebrae, the bone and marrow tissue volume (TV, μm^2), bone tissue area (BA, μm^2), bone surface perimeter (BS, μm), Cn bone volume (BV, μm^2), percent Cn bone (BV/TV, %), trabecular bone thickness (Tb.Th, μm), trabecular separation (μm), and trabecular number (#/mm) were measured.

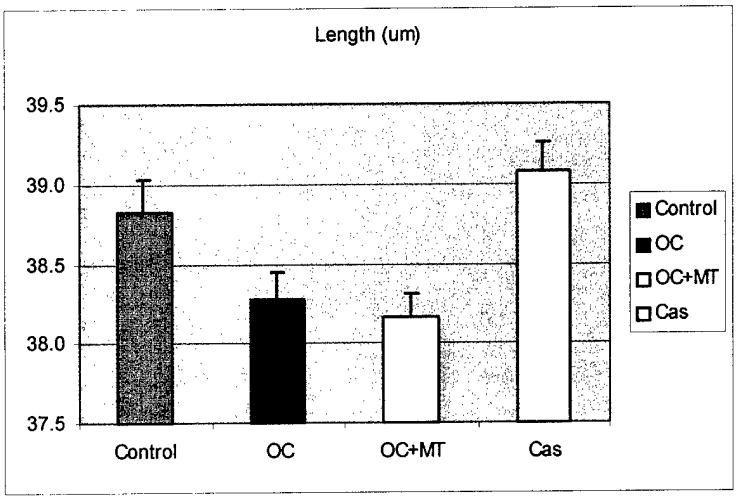
Statistics Data were tested for normality. Levene's test was used to evaluate homogeneity of variances. If data was not normal and if variances were not homogeneous, non-parametric tests (Chi-square and Kruskall-Wallis tests) were used for comparisons. Normally distributed data was analyzed by Analysis of variance (ANOVA) and Post hoc analyses were conducted using Tukey's honest significance test. In addition, and for comparison purposes, t-tests for independent samples were conducted against Control (OC vs. Control, OC+MT vs. Control, Anti-Androgen vs. Control), and between OC groups (OC vs. OC+MT).

Results

General No significant histopathologic findings were noted. The results presented here are a synopsis of the length and histomorphometric findings.

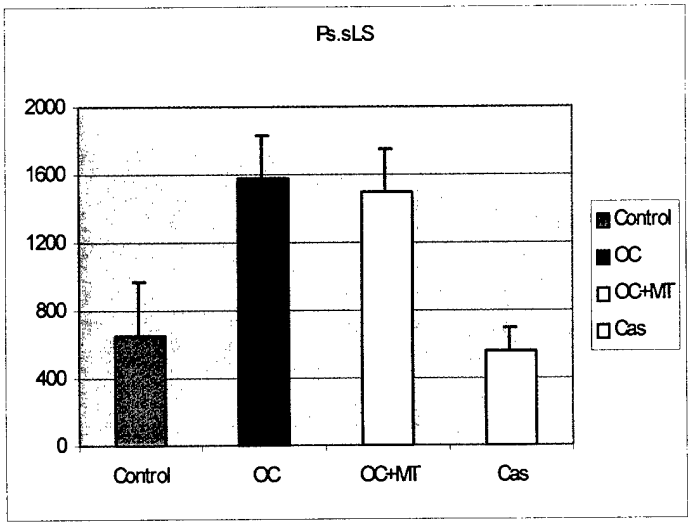
Length

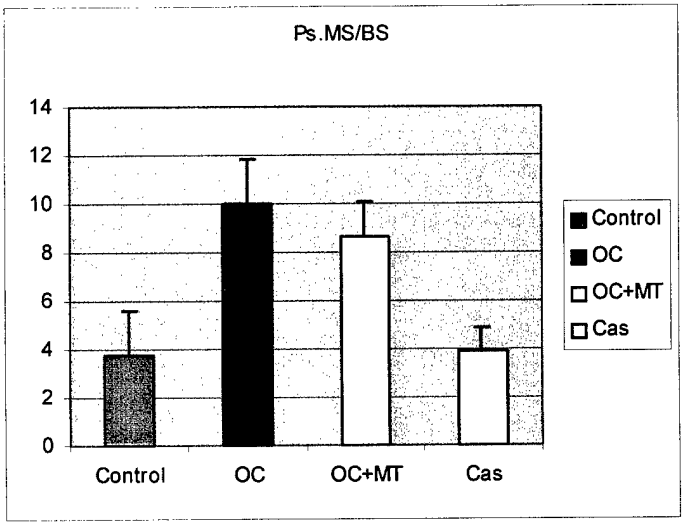
Treatment with OC's, ± MT, suppressed the longitudinal growth. By Tukey's HSD test, OC+MT treated rats had shorter tibias that Control rats ($p = 0.055$). Also, OC and OC+MT treated animals had significantly shorter tibias than the Anti-Androgen treated animals ($p= 0.0186$ and $p= 0.006$, respectively). OC treatment was not different than OC+MT.



Cortical Measurements at Tibio-Fibular Junction

Significant ($p<0.05$) findings were only seen for periosteal single labeled surface (Ps.sLS; $p = 0.009$) and periosteal mineralizing surface bone surface referent (Ps.MS/BS; $p = 0.011$). OC treatment ± MT caused an increase in the amount of periostal single labeled surface. Similarly, OC treatment with or without MT caused an increase in the amount of periostal single mineralizing surface.

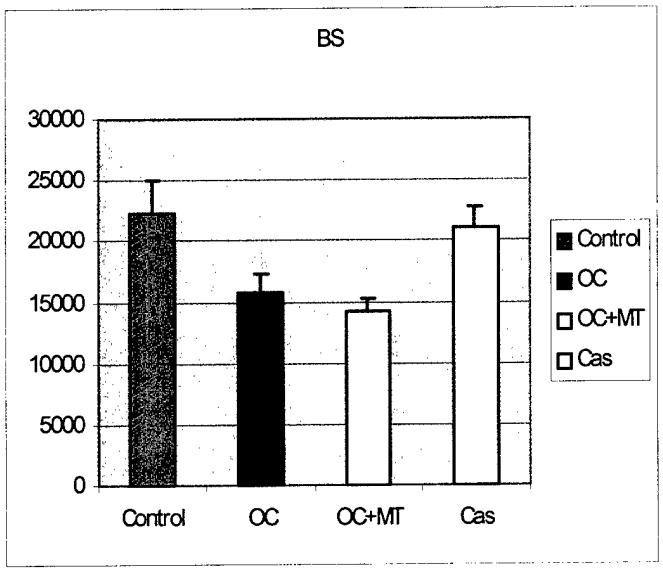


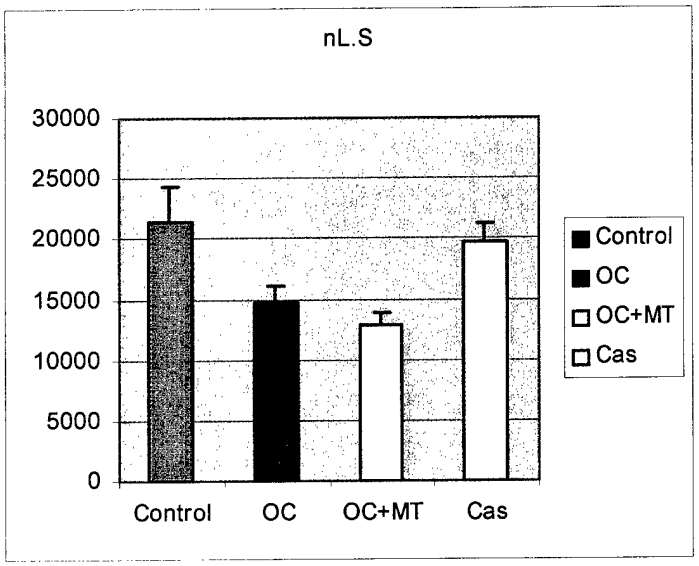


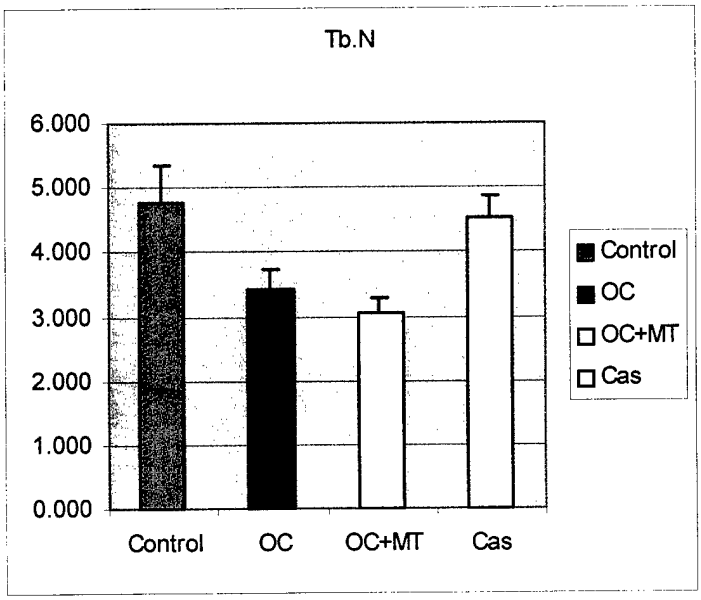
Cancellous Measurements at Proximal Tibia

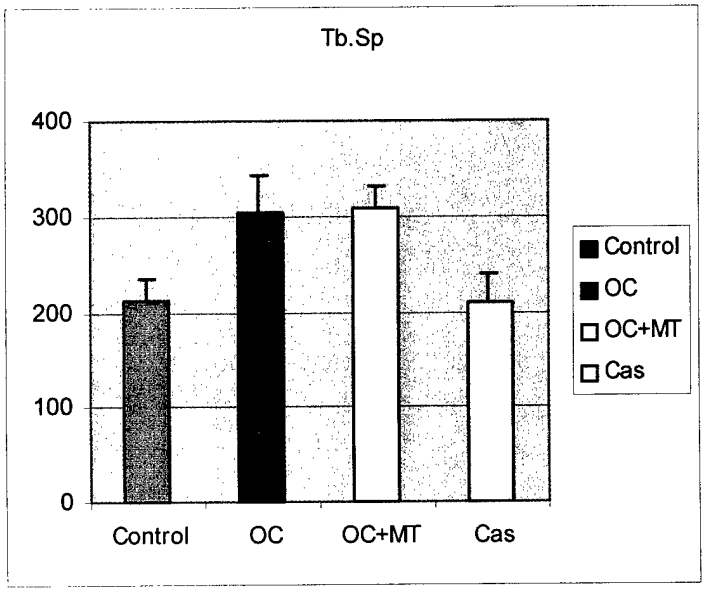
Most of the changes observed were found at the cancellous bone of the proximal tibia. The mineralizing surface bone surface referent was different ($p = 0.051$) among groups. Addition of MT to the OC treatment caused a shift in the amount of mineralizing surface. The OC group was significantly lower than the OC+MT group ($p<0.01$).

Bone surface, non-labeled surface, trabecular number, trabecular separation, bone formation rate surface referent and the amount of double labeled surface were all significantly different among the groups (ANOVA p values = 0.005, 0.005, 0.007, 0.021, 0.022, and 0.049, respectively).

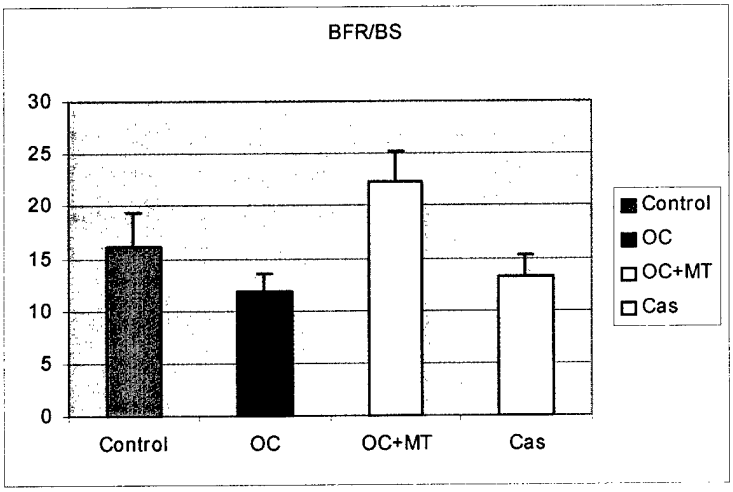


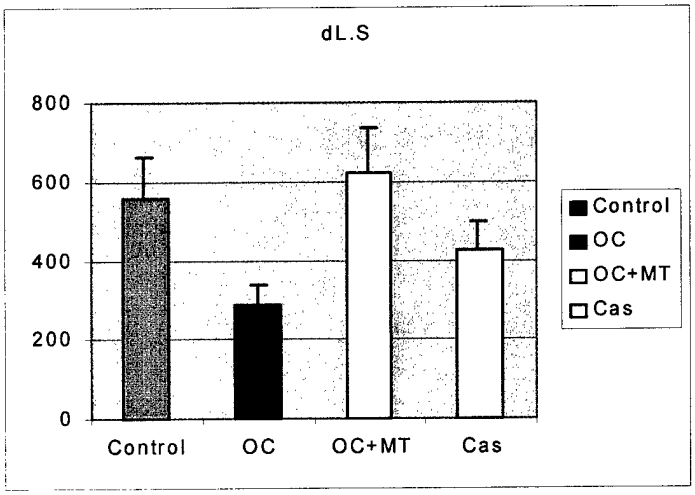






As seen in the following figures, addition of MT to the OC treatment caused a significant ($p<0.05$) increase in BFR/BS and dLS suggestive of increased bone formation. The OC+MT group was not different from the Control group.





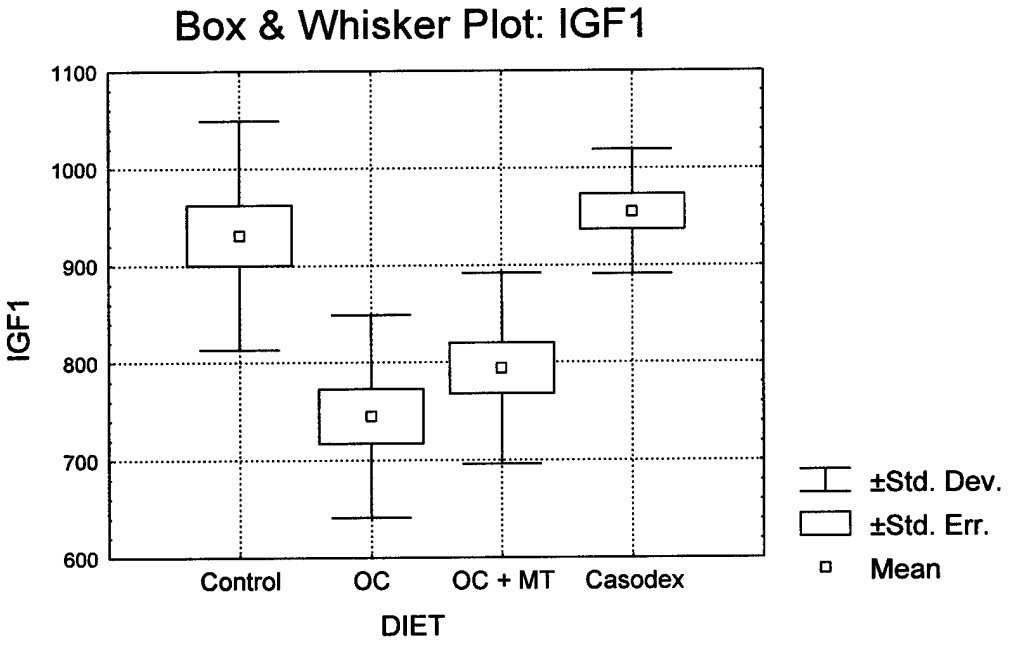
Cn Measurements of L3 Vertebra

No significant findings were found among groups for any parameter measured.

Effects of OC treatment on Serum Insulin-Like Growth Factor I

Several lines of evidence suggest that insulin-like growth factors, in particular IGF-1, are important in the regulation of bone and cartilage growth and appear to be involved in the attainment of peak bone mass. IGF-1 production is dependent upon both growth hormone and sex hormones, although the exact relationships between these factors is not completely defined. Growth hormone deficient patients have low serum IGF-I which becomes elevated (as does bone mass) following GH replacement therapy (Bing-You et al. 1993, Jorgensen et al. 1989). Circulating IGF-I levels have been shown to be significantly correlated with bone density in post-menopausal women (Boonen 1996, Mohan 1995) . Finally, IGF-I levels in serum and the skeleton have been shown to decrease with age, as do androgens. Circulating IGF-I provide a reasonable estimate of GH secretion, which is under the regulation gonadal steroids. Thus, reductions in IGF-I may result as a consequence of OC use (perhaps via androgen supression), providing a central mechanism for inhibition of bone density increases by OC use. Serum IGF-I concentrations were measured using a specific assay for rat IGF-I which involves an initial extraction of the serum to remove interfering IGF-binding proteins (DSL, Webster Tx).

I. Effects of OC on Serum IGF-1 in Sprague Dawley Rats



Results: Serum IGF-1 levels were significantly different between groups ($F[3,50]=14.3$, $p=0.00001$). Serum IGF-1 was suppressed in the OC ($p<0.0002$) and OC+MT ($p<0.003$)treatment groups relative to the Control group. This effect was not reversed by addition of MT to the OC regimen, as OC and OC+MT groups were not different from one another ($p=0.56$). Anti-androgen administration did not effect serum IGF-1 levels ($p=0.93$).

II. In Cynomolgus Monkeys Treated with OC from the previous project providing the basis for the current study.

Additional studies were carried out to determine if the findings in the present study regarding effects of OC treatment on serum IGF1 and markers of bone metabolism were also observed in non-human primates. For these studies we utilized banked serum samples collected at necropsy from an NIH funded study to examine the effects of individual components of triphasic oral contraceptives on the development of coronary artery

atherosclerosis (Adams et al., 1997). The experiment was designed to determine the combined and individual effects of ethinyl estradiol and levonorgestral, the two components of the oral contraceptive regimen used in the original study by Register et al., 1997, and in the present study in the rats. One group served as placebo-treated controls (n = 20). A second group received OC in the form of ethinyl estradiol (E2) (Wyeth-Ayerst Research, Princeton, NJ) and levonorgestrel (Wyeth-Ayerst Research) (n = 20). A third group received ethinyl E2 only (n = 21). A fourth group received levonorgestrel only (n = 20). The hormones were mixed in the diet and animals in groups two, three, and four were treated on a 28-day triphasic schedule. The schedule was days 1 to 6, 8.2 g levonorgestrel and/or 5 g ethinyl E2; days 7 to 11, 12.5 g levonorgestrel and/or 6.7 g ethinyl E2; days 12 to 21, 21 g levonorgestrel and/or 5 g ethinyl E2; and days 22 to 28, inert placebo. As in previous experiments, menstrual cyclicity ceased in contraceptive steroid-treated monkeys. We hypothesized that serum osteocalcin levels would be suppressed by oral contraceptive treatment as previously observed by Register et al. and that serum IGF-1 levels would be suppressed as in the present study in rats. Suppression of serum IGF1 in both studies would provide support for the idea that suppression of central hypothalamic- growth hormone- IGF1 axis could be a major mechanism by which bone metabolism would be impaired by OCs.

Results and Conclusions

Our initial data from the non-human primate study did not confirm our previous findings in the non-human primate or the rat with respect to osteocalcin or IGF-1 (data not shown). Subsequent reexamination of the circumstances surrounding collection of serum at necropsy revealed that the individual experimental groups were handled differently at necropsy. As such we have concluded that additional testing of serum obtained from timepoints prior to necrospsy, when all conditions were equivalent, is necessary for firm conclusions regarding the effects of these treatments on serum IGF1. We plan to carry out those studies in the coming months.

Additional Plans for Completion of the Project

1) Determination of Serum Biomarkers of Bone Resorption: As of September, 2001, a new assay for determination of serum collagen crosslink concentrations in rats (Ratlaps®) has become available from Osteometer Biotech A/S (Herlev, Denmark) which we plan to use for assessment of rat bone resorption parameters.

2) Determination of Serum IGF-1 and Bone Metabolism Markers in Non-Human primates treated with individual OC components.

3) Direct biochemical analysis of tissues from the rat study as feasible.

4) Analyze Additional Data and Prepare manuscript(s) for publication

5) Submit manuscript(s), revise and resubmit as necessary.

KEY RESEARCH ACCOMPLISHMENTS

The key findings of the study are:

1) OC use decreased the peak bone mass of young intact female rats, similar to the findings in cynomolgus monkeys.

2) Addition of a non-aromatizable androgenic steroid to OCs, at the dose provided, did not prevent the adverse effects of OCs to the growing skeleton of young rats.

3) Anti-androgen treatment did not cause an adverse effect on the growing skeleton of young rats at the achieved dose, contrary to the hypothesized effects.

4) Histomorphometric data suggest that OC use suppressed bone turnover.

5) Addition of MT to OC appeared to increase bone formation.

6) Effects of OC on bone density were paralleled by alterations in serum IGF-1 in the rats, suggesting a central mechanisms underlying detrimental effects of OC on bone mineral accretion.

REPORTABLE OUTCOMES
Oct 2000-Sept 2001

Abstract presented at the 2001 Meeting of the Endocrine Society

Register, TC and Jayo, MJ. Oral contraceptive inhibition of bone growth in young female rats: the role of androgens. Proceedings of the 83rd Annual Meeting of the Endocrine Society, P2-361, June 2001.

Abstract Preview for Abstract 41671

ORAL CONTRACEPTIVE INHIBITION OF BONE GROWTH IN YOUNG FEMALE RATS: THE ROLE OF ANDROGENS .

T.C. Register[1] and M.J. Jayo[1,2]. [1]Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, NC, United States, [2]Pathology Associates, Charles River Laboratories, Advance, NC, United States

Oral contraceptives (OC) have been shown to inhibit increases in whole body and lumbar spine bone mineral content and density in intact young adult female monkeys (Register et al., Osteoporosis Intl 1997;7:348). OC treatment also caused a marked suppression of serum testosterone and androstenedione levels. The purpose of the present study was to determine whether hypoandrogenemia might explain the inhibitory effects of OC on bone metabolism and the attainment of peak bone mass in females. Intact, 70-day old adolescent/young adult virgin female Sprague-Dawley rats were treated with placebo (Control), an OC containing ethinyl estradiol and levonorgestrel (OC), OC supplemented with the aromatization-resistant androgen methyltestosterone (OC+MT), or the anti-androgen bicalutamide (BC) for 15 weeks. OC treatment inhibited gains in bone mass relative to Controls, similar to the previous findings in cynomolgus monkeys, and also caused a reduction in tibial length. Addition of MT to the OC treatment did not prevent the adverse effects of OCs on the bone mass or tibial length, suggesting that hypoandrogenemia was not solely responsible for the OC effects. Anti-androgen (BC) treatment had no effect on the growing skeleton of young rats compared to Controls, suggesting that androgens may have a limited involvement in acquisition of bone mass in young adult females. Histomorphometry of the cancellous compartment of proximal tibias indicated the OC and OC+MT groups had reduced bone surface, non-labeled surface, and trabecular number, and increased trabecular separation relative to Control and BC groups. Interestingly, histomorphometric data suggested that double-labeled surface perimeter, bone formation rate/bone surface ratio, and mineralizing surface were higher in the OC+MT group compared to the OC group. Nevertheless, OC and OC+MT groups were not different from one another in bone density or tibial lengths. No treatment effects on the cortical compartment were observed. Taken together, these results suggest that while androgens may influence bone metabolism, hypoandrogenemia was not the underlying cause of OC inhibition of bone mineral accretion in this study.

Abstract presented at the 2001 American Society for Bone and Mineral Research Meeting

Reference: Register, TC and Jayo, MJ. Oral contraceptive inhibition of bone growth in young female rats: the role of androgens. J Bone Miner Res 2001;16(Suppl 1):S317.

Oral Contraceptive Inhibition of Bone Growth in Young Female Rats: the Role of Androgens

T. Register,1 M. J. Jayo.21Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, NC, USA, 2Pathology Associates, Charles River Laboratories, Advance, NC, USA.

Previous studies demonstrated that treatment of intact young adult female monkeys with an oral contraceptive (OC) resulted in inhibition of normal gains in whole body and lumbar spine bone mineral content and density (Register et al., Osteoporosis Intl 1997;7:348). OC treatment also caused a marked suppression of serum testosterone and androstenedione levels. The purpose of the present study was to determine whether hypoandrogenemia might explain the inhibitory effects of OC on bone metabolism and the attainment of peak bone mass in young adult females. Intact, 70-day old adolescent/young adult virgin female Sprague-Dawley rats were treated with placebo (Control), an OC containing ethinyl estradiol and levonorgestrel (OC), OC supplemented with the aromatization-resistant androgen methyltestosterone (OC+MT), or the anti-androgen bicalutamide (BC) for 15 weeks. OC treatment inhibited gains in bone mass relative to Controls, similar to the previous findings in cynomolgus monkeys, and also caused a reduction in tibial length. Addition of MT to the OC
treatment did not prevent the adverse effects of OCs on the bone mass or tibial length, suggesting that hypoandrogenemia was not solely responsible for the OC effects. Anti-androgen (BC) treatment had no effect on the growing skeleton of young rats compared to Controls, suggesting that androgens may have a limited involvement in acquisition of bone mass in young adult females. Histomorphometry of the cancellous compartment of proximal tibias indicated that the OC and OC+MT groups had reduced bone surface, non-labeled surface, and trabecular number, and increased trabecular separation relative to Control and BC groups. Histomorphometric data suggested that double-labeled surface perimeter, bone formation rate/bone surface ratio, and mineralizing surface were higher in the OC+MT group compared to the OC group. Nevertheless, OC and OC+MT groups were not different from one another in bone density or tibial lengths. No treatment effects on the cortical compartment were observed. Taken together, these results suggest that while androgens may influence bone metabolism, hypoandrogenemia was not the underlying cause of OC inhibition of bone mineral acquisition in this study.

CONCLUSIONS

The results from this project suggest that OCs may inhibit bone metabolism and the acquisition of peak bone mass in rats, in part confirming the previous finding in cynomolgus macaques (Register et al., 1997). The addition of a non-aromatizable androgen (MT) to the OC did not significantly counteract the effects of OC treatment on bone mass and the anti-androgen bicalutamide did not mimic the effects of OC treatment on bone density or metabolism, suggesting that hypoandrogenemia does not play a central role in the effects of OC on bone in young adult females. Androgens, natural or synthetic, are not part of any OC therapy available to women, and to our knowledge, this is the first time that the effects of addition of androgens a low-estradiol containing OCs with or without on bone tissues of skeletally immature and reproductively sound subjects have been evaluated.

The histomorphometric data demonstrated that OC treatment with or without MT caused significant osteopenia at the proximal tibia cancellous bone, with increased tabecular separation and a decrease in the trabecular number. In addition, double labeled surface and the BFR/TV were significantly ($p<0.03$) lower in the OC-treated animals compared to Controls. These findings suggest a mild suppression of bone turnover in the OC treated animals compared to Controls. It should be noted that in this study OC treatment alone did not alter circulating markers of bone formation ALP and osteocalcin (which had been significantly suppressed in OC-treated monkeys), suggesting species differences in the response to OC. It is possible that changes in circulating osteocalcin with age overwhelmed any suppressive effect of OC treatment. Peak circulating levels of osteocalcin in the rat are found at about 21 days of age and rapidly and significantly decrease to a nadir by 16 weeks of age (Liu and Lin). Our findings were similar to those previously reported. Alternatively, this could be indicative of a dose effect since the rats received 30% less than the non-human primates based on the human dose.

MT supplementation to OC treatment had no effect on BMC or BMD relative to OC treatment alone, although certain bone metabolic and histomorphometric parameters were effected. For example, addition to OCs of MT significantly suppressed osteocalcin levels relative to the OC group. Histomorphometrically, BFR/BS, MS/BS, dL.S, and BFR/BV were all significantly higher (<0.03) in the OC+MT group compared to the OC only treated animals. It is not known if dose modification would provide beneficial effects to the skeleton, although it is worth noting that addition of MT to OC also caused liver effects as demonstrated by elevations of serum ALT. These liver effects were not seen grossly (see liver weight bar graph) or histologically (not presented).

Young women who take OCs suppress endogenous formation and serum levels of bioavailable sex hormones, by direct and central negative feedback and by indirectly affecting the circulating levels of SHBG. Consequently, the level of bioavailable androgen and estrogen at the tissue level may be modulated by OCs. Effects at other hormone sensitive organs (endometrium and mammary gland) are also likely.

Interestingly, the osteopenic effect caused by OC or OC+MT treatment at the cancellous bone may have led to a self-protective bone re-distribution (related mechanostat and biomechanical stress). Evidence for this is the increased bone formation at the cortical surfaces, as seen by the increase in single label and mineralization surfaces on the cortical periosteum.

Confounds to the present study

The results obtained in this study, at least as far at the effects of OC treatment, are somewhat confounded by the failure of the rats to consume their diets containing the OC. The differences in diet consumption led to differences in body weight, which is generally associated with bone mass and density. Such alterations in diet consumption were not observed in the previous study (Register et al., 1997) in cynomolgus monkeys which

served as the stimulus for this project. Nevertheless, the addition of the non-aromatizable androgen to OC treatment did not affect diet consumption relative to the OC only group, neither did the addition of the androgen antagonist relative to the control group receiving no hormone therapy. The finding that the rats in this study did not eat equivalently the diets containing the hormones has some precedent, despite our pilot studies which suggested otherwise. Manoharan, et al (1970) used diet as the method for OC delivery which led to less food consumption and lower BW. Interestingly, SQ injections of OC also have led to reductions in BW (Lea et al., 1996). Regardless as to cause, lack of appetite and/or food aversion, BW were significantly reduced in the OC and OC+MT groups. It is difficult to determine the absolute role that alterations in BW play in the effects of the OC and OC+MT treatment on the skeleton. BW at scan time 12 weeks correlated positively and significantly ($p<0.05$) with spine BMD and pQCT TBMD at the same time point ($r=0.566$ and 0.429, respectively). However, the amount of diet and drug consumed was sufficient to provide for measurable differences in circulating sex hormones, and liver and bone biomarkers.

In summary, although interpretation is complicated by the BW effects, our findings support the previous finding that OC use by young individuals appears to prevent proper bone accrual and maximal peak bone mass (PBM) (5-7). OCs, at the dose and route given, negatively affected acquisition of PBM and skeletal integrity in young rats. Supplementation of OCs with androgens, in the dose and form of MT, failed to prevent the OC-induced bone effect. Use of the anti-androgen Casodex®, at the dose provided, did not cause adverse skeletal effects. Overall the data do not support a role for hypoandrogenemia in the inhibitory effects of OC on bone mineral acquistition in young adult animals. In effect, androgen supplementation may not be adequate to prevent adverse effects of OC on the skeleton. Initial findings regarding the reduction of serum IGF-1 levels suggest an inhibition of GH secretion via the HPA axis may underly some of the OC effects. Additional studies into the mechanism by which OCs influence normal bone acquisition are necessary before full understanding of the effects are achieved.

REFERENCES

Adams MR, Anthony MS, Manning JM, et al. Obstet Gynecol. 2000;96(2):250-5.
Bing-You RG, Denis MC, Rosen CJ. Calcif Tissue Int. 1993;52(3):183-7
Boonen S, Lesaffre E, Dequeker J, Aerssens J, et al. J Am Geriatr Soc. 1996;44(11):1301-6.
Boonen S, Lesaffre E, Aerssens J, Pelemans W, et al. Gerontology. 1996;42(6):330-8.
Gasser JA. Bone 17(4):145S-154S, 1995.
Jorgensen JO, Pedersen SA, Thuesen L, Jorgensen J, et al. Lancet. 1989;1(8649):1221-5.
Kreipe RE, Hicks DG, Rosier RN, et al. J Adolescent Health. 14(4):319-24, 1993.
Lea C, Kendall N, Flanagan AM. Calcif Tissue Int 58:268-272, 1996.
Liu FTY, Lin HS. PSEBM. 133(4):1354-7, 1970
Manoharan K, Yang MG, Mickelsen O. PSEBM. 133(3):774-9, 1970
Modrowoski D, del Pozo E, Miravet L. Horm Metab Res 24(10):474-477, 1992.
Mohan S, Farley JR, Baylink DJ. Prog Growth Factor Res. 1995;6(2-4):465-73.
Parfitt AM, Drezner MK, Glorieux FH, et al. J Bone Mineral Res 1987;2:595-610.
Polatti F, Perotti F, Filippa N, et al. Contraception. 51(4):221-4, 1995.
Register TC, Jayo MJ, Jerome CP. Osteoporosis Intl. 7(4):348-53, 1997.
Sato M. Bone 17(4):157S-162S, 1995.

WAKE FOREST
UNIVERSITY
SCHOOL of MEDICINE
Comparative Medicine Clinical Research Center

Oral Contraceptive Inhibition Of Bone Growth In Young Female Rats: The Role Of Androgens.

Thomas C Register PhD[1] and Manuel J Jayo DVM, PhD[1,2]
[1]Pathology/Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, NC
[2]Pathology Associates, A Charles River Company, Advance NC.

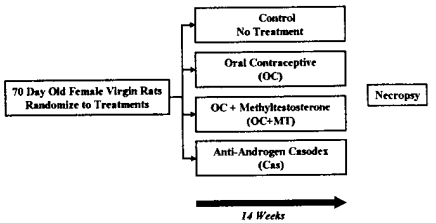
ABSTRACT

ORAL CONTRACEPTIVE INHIBITION OF BONE GROWTH IN YOUNG FEMALE RATS: THE ROLE OF ANDROGENS . T.C. Register1 and M.J. Jayo1,2.

Oral contraceptives (OC) have been shown to inhibit increases in whole body and lumbar spine bone mineral content and density in intact young adult female monkeys (Register et al., Osteoporosis Intl 1997;7:348). OC treatment also caused a marked suppression of serum testosterone and androstenedione levels. The purpose of the present study was to determine whether hypoandrogenemia might explain the inhibitory effects of OC on bone metabolism and the attainment of peak bone mass in females. Intact, 70-day old adolescent/young adult virgin female Sprague-Dawley rats were treated with placebo (Control), an OC containing ethinyl estradiol and levonorgestrel (OC), OC supplemented with the aromatization-resistant androgen methyltestosterone (OC+MT), or the anti-androgen bicalutamide (BC) for 15 weeks. OC treatment inhibited gains in bone mass relative to Controls, similar to the previous findings in cynomolgus monkeys, and also caused a reduction in tibial length. Addition of MT to the OC treatment did not prevent the adverse effects of OCs on the bone mass or tibial length, suggesting that hypoandrogenemia was not solely responsible for the OC effects. Anti-androgen (BC) treatment had no effect on the growing skeleton of young rats compared to Controls, suggesting that androgens may have a limited involvement in acquisition of bone mass in young adult females. Histomorphometry of the cancellous compartment of proximal tibias indicated the OC and OC+MT groups had reduced bone surface, non-labeled surface, and trabecular number, and increased trabecular separation relative to Control and BC groups. Interestingly, histomorphometric data suggested that double-labeled surface perimeter, bone formation rate/bone surface ratio, and mineralizing surface were higher in the OC+MT group compared to the OC group. Nevertheless, OC and OC+MT groups were not different from one another in bone density or tibial lengths. No treatment effects on the cortical compartment were observed. Taken together, these results suggest that while androgens may influence bone metabolism, hypoandrogenemia was not the underlying cause of OC inhibition of bone mineral accretion in this study.

INTRODUCTION

Oral contraceptives (OC) significantly inhibit normal bone acquisition in intact young adult female monkeys (1). The OC effect on bone mineral accrual may be due to hypoandrogenemia, a well-known side effect caused by OC use. This experiment was designed to test if androgen supplementation during OC use may prevent the inhibition of bone mass acquisition in young subjects.



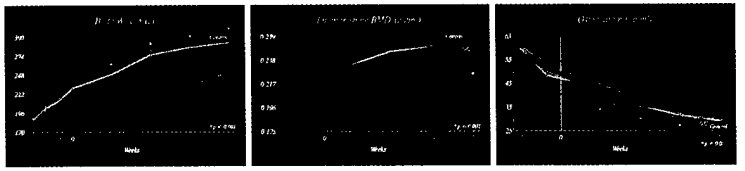
DESIGN

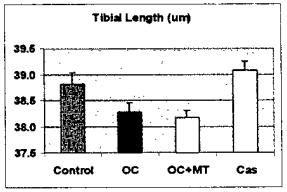
Seventy-day-old intact virgin female Sprague-Dawley rats were randomized to four groups based on body weight (BW) and lumbar spine bone mineral density (BMD g/cm^2) by DEXA. At 70 days of age (227 g), groups were treated with or without drugs mixed in their diet for 15 weeks: (1) ***Control***; (2) ***OC*** (Levlen®, levonorgestrel + ethinyl- estradiol at 0.0310 mg and 0.00619 mg per 100 g of diet, respectively); (3) OC + Android® (***OC+MT***, ICN Pharmaceuticals, Inc, methyltestosterone at 0.516 mg/100 g), and (4) Casodex® (Zeneca Pharmaceuticals, ***Cas***, an antiandrogen at 10.33 mg/100 g). These doses were prepared to mimic a woman's contraception (0.5 mg LGN+0.03 mg EE) and postmenopausal hormone replacement (2.5 mg MT), and a man's anticancer (50 mg Cas) daily dose based on 1800 cal/day. Food consumption and BW were measured daily. ***OC*** and ***OC+MT*** groups received steroids for 3 consecutive days and base diet on the 4th day (similar to a woman's four week estrous and pill cycle). Animals in the ***Cas*** group received the antiandrogen-containing diet every day. Animals in the ***OC*** and ***OC+MT*** groups were sampled and killed on days 2 or 3 of the drug cycle.

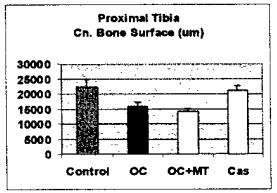
RESULTS

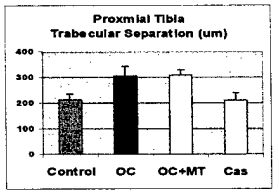
Body Weight, Bone Density, and Bone Biomarkers

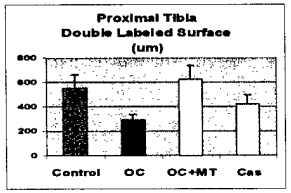
All groups gained significant ($p<0.05$) BW and spinal BMD through time. ***Control*** and ***Cas*** animals gained more BW and BMD than ***OC*** and ***OC+MT*** groups ($p<0.05$). Serum Osteocalcin and ALP (data not shown) significantly decreased ($p<0.05$) with time in all four groups, consistent with an age dependent decline in these markers. ***OC+MT*** had lower levels of osteocalcin (bone effects) than ***Control*** and ***OC*** groups.

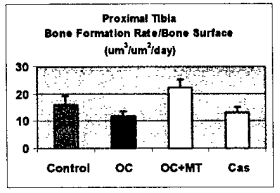












SUMMARY

OC treatment inhibited gains in bone mass relative to Controls, similar to the previous findings in cynomolgus monkeys, and also caused a reduction in longitudinal growth (tibial length) and body weight.

Addition of MT to the OC treatment did not prevent the adverse effects of OCs on the bone mass or tibial length, suggesting that hypoandrogenemia was not solely responsible for negative effects of OC.

Anti-androgen (BC) treatment had no effect on the growing skeleton of young rats compared to Controls, suggesting that androgens may have a limited involvement in acquisition of bone mass in young adult females.

Histomorphometry of the cancellous compartment of proximal tibias indicated the OC and OC+MT groups had reduced bone surface, non-labeled surface, and trabecular number, and increased trabecular separation relative to Control and BC groups.

Histomorphometric data suggested that double-labeled surface perimeter, bone formation rate/bone surface ratio, and mineralizing surface were higher in the OC+MT group compared to the OC group. Nevertheless, OC and OC+MT groups were not different from one another in bone density or tibial lengths.

No treatment effects on the cortical compartment were observed.

Taken together, these results suggest that while androgens may influence bone metabolism, hypoandrogenemia was not the underlying cause of OC inhibition of bone mineral accretion in this study.

DISCUSSION

OCs, at the dose and route given, negatively affected acquisition of PBM and skeletal integrity in young rats. Although interpretation is complicated by the BW effects (8), these data support the previous finding that OC use by young individuals appears to prevent normal bone accrual and increases in bone density (1,5,6). Supplementation of OCs with androgens, in the dose and form of MT, failed to prevent the OC-induced bone effect, although increases in bone formation rate relative to OC were observed histomorphometrically despite a reduction in serum osteocalcin. Effects of treatments on markers of bone resorption have not yet been determined. Use of the anti-androgen Casodex®, at the dose provided, did not cause adverse skeletal effects. Bone deficits have been reported in rats at a Casodex® dose of 25 mg/kg (7), which was approximately 3 times higher to ours. In the OC-treated rats, serum ALP and osteocalcin levels, which were previously shown to be significantly suppressed in OC-treated monkeys (1), were not affected, suggesting species differences in the response to OC. More robust determination of the potential effects of androgen supplementation may require reevaluation in a primate.

BIBLIOGRAPHY

1. Register TC, Jayo MJ, Jerome CP. Osteoporosis Intl. 7(4):348-53, 1997.
2. Manoharan K, Yang MG, Mickelsen O. PSEBM. 133(3):774-9, 1970
3. Liu FTY, Lin HS. PSEBM. 133(4):1354-7, 1970
4. Modrowoski D, del Pozo E, Miravet L. Horm Metab Res 24(10):474-477, 1992.
5. Polatti F, Perotti F, Filippa N, et al. Contraception. 51(4):221-4, 1995.
6. Kreipe RE, Hicks DG, Rosier RN, et al. J Adolescent Health. 14(4):319-24, 1993.
7. Lea, C Kendall N, Flanagan AM. Calcif Tissue Int 58:268-72, 1996.
8. Jayo MJ, Register TC, Hughes CL. et al. J Soc Gyn Invest7:257-265, 2000.

Acknowledgement
This project was supported in part grant number DAMD117-98-1-8514 of the US Department of Defense.
Some concepts presented here are part of US Patent 5,770,226 property of WFUSM.